Transformation of *Lilium Longiflorum* Plants for Cucumber Mosaic Virus Resistance by Particle Bombardment

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Abstract

***Lilium* species (Liliaceae) are a significant floriculture commodity and one of the three major bulb crops in the commercial market. Lilies are monocotyledonous plants that have been generally recalcitrant to molecular genetic manipulation because of limitations that restrict utilization of transformation technologies that are routinely applied to dicotyledonous plants. Cucumber mosaic virus (CMV) causes a serious disease of lily. It is known that transgenic plants bearing a disabled CMV replicase gene can be resistant to that virus. About 5000 pieces of morphogenic calli, 3-4 mm in diameter, derived from segments of sterile bulblet scales of *L. longiflorum* Thunb. cv. 'Snow Queen' were microprojectile bombarded, using a Finer-type of bombardment apparatus, followed by bialaphos selection. The plasmid p35SAc containing PAT selectable marker gene encoding phosphinothricin-N-acetyltransferase (that detoxifies the herbicide "Basta") under the control of 35S promoter and the nopaline synthase (NOS) terminator; and the plasmid pSAN101 containing the CMV defective replicase gene under the control of Act promoter and NOS terminator, were used. After several rounds of callus selection, plants were regenerated in vitro. PCR analysis indicated that these plants contain both the PAT and the disabled CMV replicase transgenes.**

INTRODUCTION

Lilium species (Liliaceae) are a significant floriculture commodity and one of the three major bulb crops in the commercial market (Robinson and Firoozabady 1993). Lilies, like other important floral bulb crops, are monocotyledonous plants, which have been generally recalcitrant to molecular genetic manipulation because of restrictions of transformation technologies that are routinely applied to dicotyledonous plants. The recent development of microprojectile bombardment systems (Klein et al., 1988; Sanford, 1988) and their demonstrated utility for DNA delivery into plant cells with high morphogenic capacity presents an opportunity for transformation of monocotyledonous floriculture crops (Sanford et al., 1993).

During the last decade, particle bombardment has been used to obtain transgenic plants of floriculture crops such as tulip (Wilmink et al., 1992), orchid (Kuehnle and Sugii 1992), gladiolus (Kamo et al., 1995) and lily (Langeveld et al., 1995; Watad et al., 1998). However, there are no reports that document recovery of transgenic *L. longiflorum* plants bearing genes that may confer virus resistance.

We report here the recovery of transgenic lily plants after microprojectile bombardment of morphogenic primary callus with plasmids containing *uidA* reporter and *PAT* selectable marker genes and the plasmid pSAN101 containing the CMV defective replicase gene. Plants were regenerated under bialaphos selection. The presence of the transgenes in regenerated plants was determined by polymerase chain reaction (PCR) amplification.

MATERIALS AND METHODS

Plant Material and Tissue Culture of Morphogenic Callus

Bulblets were derived in vitro from cultured inner scale explants of *L. longiflorum* Thunb. 'Snow Queen' bulbs. Two to 4 mm segments of the bulblets were excised and cultured on meristem initiation medium. The medium contains MS salts (Murashige and Skoog 1962), supplemented with 0.4 mg/l thiamine HCl, 100 mg/l myo-inositol, 30 g/l sucrose, 0.2 mg/l NAA, and 5 g/l Difco Bacto agar, pH 5.7-5.8. For callus induction and subcultivation of friable callus, the medium comprised MS salts and basal organic constituents, supplemented with 10 mg/l thiamin HCl, 0.1 mg/l BA, 30 g/l sucrose, 1 mg/l NAA and 5 g/l Difco Bacto agar, pH 5.7-5.8. When BA was replaced with thidiazuron (TDZ) 1 mg/l in this medium, hard compact callus was obtained. For shoot regeneration, medium contained MS salts and basal organic constituents supplemented with 10 mg/l thiamine HCl, 30 g/l sucrose, 1 mg/l BA, 0.1 mg/l NAA, and 5 g/l Difco Bacto agar, pH 5.7-5.8, was used.

Transformation Vectors and Microprojectile Bombardment

The plasmid pUBQ3genGUS, containing the *uid*A reporter gene encoding β-glucuronidase (GUS) under the control of the *Arabidopsis* ubiquitin promoter, was kindly provided by Dr. J. Callis (Univ. of Arizona, Tucson). The plasmid p35SAc which contains the selectable marker gene *PAT* encoding phosphinothricin- N-acetyltransferase (PAT detoxifies bialaphos, the active compound in the herbicide "Basta") under the control of the 35S promoter and the nopaline synthase (NOS) terminator, was constructed in Beltsville. The plasmid pPHP6517, which contains the *PAT* gene under the control of the maize ubiquitin promoter and the NOS terminator, was kindly provided by Pioneer Hi Bred Int. (Johnston, Iowa, USA). The plasmid pSAN101 containing the CMV defective replicase gene was kindly provided by M. Zaitlin (Cornell University, Ithaca).

Plasmid DNA for particle bombardment was isolated from *Escherichia coli* after alkaline lysis and was purified using the plasmid Midi-Kit (Qiagen Inc., Valencia, CA, USA).

Development and Optimization of the Particle Inflow Gun (PIG) for DNA Delivery into Plant Cells

A particle bombardment device (the plastic PIG) was built following the model of Finer et al. (1992), adapted according to Gray et al. (1994). All transformation experiments were conducted using the PIG. Tungsten particles M10, 0.7 µm (Bio-Rad, Richmond, CA, USA) (3 mg) were coated with 6µg of plasmid DNA (Sanford et al., 1993). For the optimization of the PIG, tungsten distribution on a filter paper (4.25 cm in diameter) was evaluated using 0.3mg tungsten particles in 4µl ethanol without DNA. Target distance from the launching plate of 18 cm, pressure of 5 bar and chamber vacuum of 70 cm Hg in the particle inflow gun (PIG) were used. Ratios of tungsten and DNA mixed in 9 µl ethanol were evaluated for impact on transformation efficiency, based on transient *uidA* expression.

Two days prior to particle bombardment, sectors of compact morphogenic calli were separated into 2-3 mm pieces and placed onto shoot regeneration medium (as described above with 1 mg/l BA and 0.1 mg/l NAA) and kept in darkness. Two days after bombardment, calli were cultured onto shoot proliferation medium containing the selection agent. Cultures were maintained under a 16 h photoperiod at 26 °C.

Selection Protocol

The herbicide bialaphos (Meiji Seika, Yokohama, Japan) was used to select callus and shoots expressing the *PAT* gene. Bialaphos was diluted to a final concentration of 0, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/l in callus maintenance or shoot regeneration media used for selection. Two days after bombardment, the calli were recultured onto shoot proliferation medium containing bialaphos and were subcultured onto fresh medium

every 2-3 weeks. Single shoots of 5 to 15 mm in height (initially arising from calli about 2-3 months after bombardment), were excised from the shoot clusters and cultured separately on selection medium.

GUS Histochemical Staining

GUS activity from *uidA* expression was assessed basically using the histochemical assay of Jefferson et al. (1987) as modified by Kosugi et al., (1990). Transient *uidA* expression was evaluated 48 h after bombardment and quantified, using a stereomicroscope, as the number of discernible blue spots per explant. No GUS activity was detected in control explants bombarded with tungsten particles without DNA.

PCR Amplification

Leaves were collected from shoots surviving on medium with 2 mg/l bialaphos and negative control shoots regenerated from unbombarded explants. DNA was isolated from the leaves according to the procedure of Fulton et al., (1995).

Approximately 500 *n*g of DNA was used as the template for PCR. Amplification of the *PAT* gene utilized the upstream primer 5'- ATG TCT CCG GAG AGG AGA CCA GTT GAG- 3' and the downstream primer 5'- GAT CTG GGT AAC TGG CCT AAC TG- 3' resulting in amplification of a fragment of about 550 bp were used. The PCR reaction was performed in 20 µl reaction volume with 1x buffer (Boehringer Mannheim), 6 mM $MgCl_2$, 0.2 mM dNTP mix, 0.25 µM of each primer and 2 units of *Taq* DNA polymerase (Boehringer Mannheim). The reaction was 90 sec at 95 °C, followed by 30 cycles of 45 sec at 94 °C, 45 sec at 55 °C and 90 sec at 72 °C with a final extension at 72 °C for 7 min.

For amplification of the defective CMV replicase gene the forward primer 5'-CAA CAT GGA AGC TAA GGT GAT GGA AC-3' and the reverse 5'-CAG ACT CGG GTA ACT CCG CCA-3' were used, resulting in amplification of a 680 bp fragment. The PCR reaction was performed in 20 µl reaction volume. The reaction was 40 sec at 94 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 68 °C and 25 sec at 74 °C with a final extension at 72 °C for 10 min. PCR products were separated in 1.5% agarose gels and were visualized with ethidium bromide.

RESULTS AND DISCUSSION

Optimization of Bombardment Parameters Based on Transient GUS Activity

For the optimization of bombardment parameters, ratio and amount of plasmid pUBQ3genGUS DNA to tungsten per shot were studied. Target distance from the filter holder of 18 cm, pressure of 5 bar and chamber vacuum of 70 cm Hg in the particle inflow gun (PIG) were used in all experiments. Different ratios of tungsten and DNA mixed in 9 µl ethanol were tested. Segments of in vitro grown green lily leaves on agar medium were bombarded. The optimal ratio of DNA and tungsten was 1/0.5 (µg/mg) in 9 µl ethanol. This ratio was used in the following experiments. The use of filter paper dampened by liquid medium as a substrate was found to be more efficient for transformation (based on GUS transient expression) than agar-solidified medium. GUS transient expression was much higher in etiolated than green leaf segments. In the experiments with the disabled CMV replicase gene segments of etiolated leaves were bombarded as well as the morphogenic calli cultures.

Selection of Transgenic Tissues (Calli and Shoots) using Bialaphos

The herbicide bialaphos was used to select callus and shoots expressing the *PAT* gene. In the preliminary selection experiments a concentration of 2mg/l was applied for callus maintenance and shoot regeneration media. When highly morphogenic callus cultures were re-established from lily mother bulbs (of the same cultivar) and when a new commercial source of bialaphos (Riedel Haen, Germany) were used for selection, the new lily cultures were found to be sensitive to lower levels of bialaphos. The bialaphos selection concentration of Watad et al. (1998), 2 mg/l, was totally lethal in the present

experiments. In order to define the optimal concentration of biolaphos for selection, a series of experiments with morphogenic callus cultures on callus maintaining and shoot regeneration medium (in darkness and under 16-hr photoperiod) containing either 0, 0.05, 0.1, 0.25, 0.5 or 1.0 mg/l bialaphos, were carried out. Lily leaf segments and highly morphogenic calli were highly susceptible to bialaphos, when the herbicide was included either in callus maintenance or shoot regeneration media. The growth and shoot regeneration of lily leaves were totally inhibited after three weeks on medium containing 1mg/l bialaphos (Fig.1). In the case of callus, the herbicide was highly toxic in light and dark growth conditions (data not shown), when it was introduced into a callus maintenance medium. Inhibition of growth was observed at 0.1 mg/l bialaphos. Concentrations of 0.25 mg/l or greater maintained for one subculture, were highly toxic. The concentration of 0.5 mg/l was chosen for selection.

Selection of Transgenic Plants using PCR

The *PAT* transgene was detected by PCR amplification in 48 of the 80 plantlets. The forty-eight transgenic plants, all regenerated from different primary calli and maintained separately after bombardment, represent 1% of the 5000 original bombarded explants.

Co-transformation with the *PAT* gene was demonstrated by PCR for 10 of 12 plantlets already shown positive for the *PAT* (Fig. 1A). The presence of the defective CMV replicase gene was also confirmed by PCR (Fig. 2B). An amplification product of approx. 680 bp was obtained with most of the putative transgenic plantlets. Although the number of independent transformation events can not be deduced from the data presented, it is clear that multiple transformation events occurred. These results confirm that the plantlets contain both the PAT and the disabled CMV replicase transgenes.

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Figures

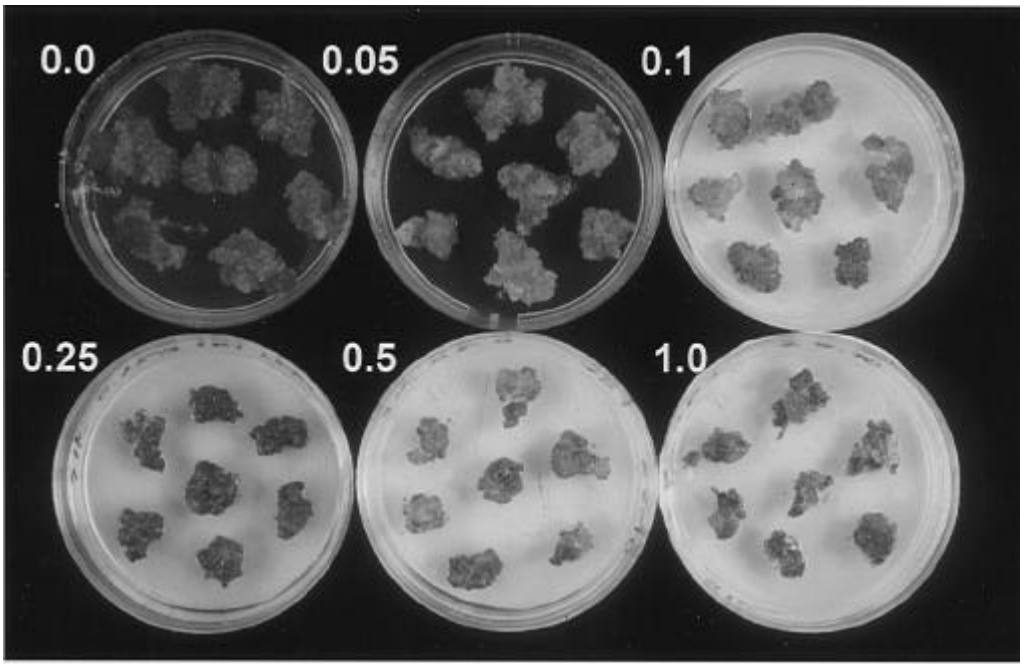

Fig.1. Response of morphogenic callus pieces to bialaphos (mg/l) after 40 days of incubation in callus maintenance medium in light.

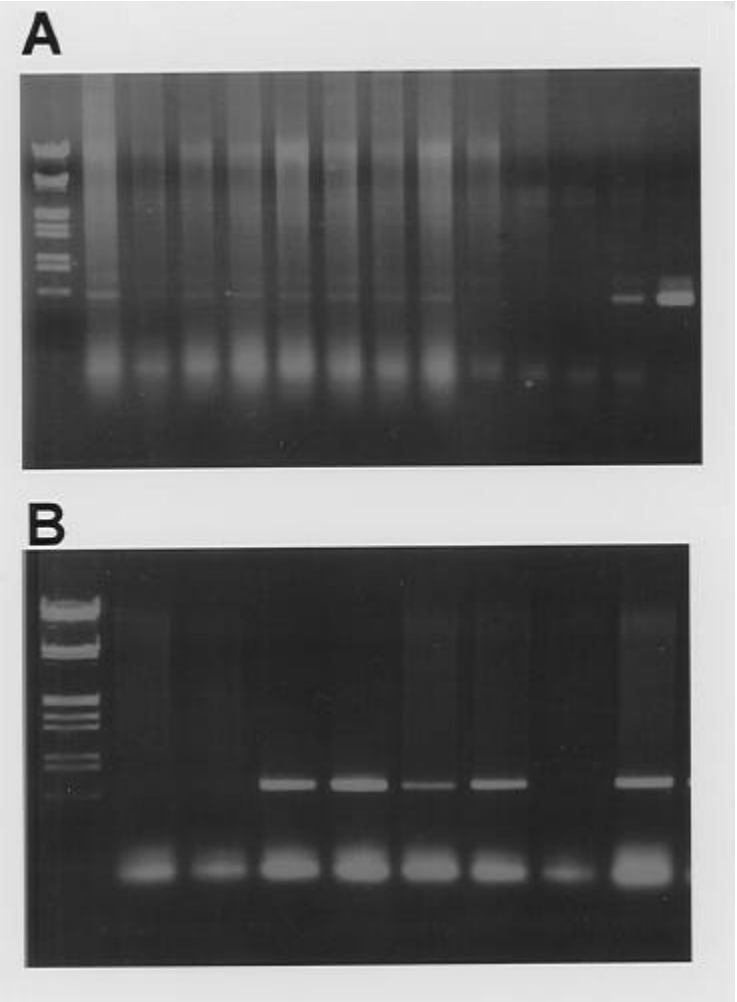

Fig. 2. PCR analysis for *PAT* (A) and defective CMV replicase genes (B) in DNA isolated from lily putative transgenic.